Starch Gel Electrophoresis of Ferns: A Compilation of Grinding Buffers, Gel and Electrode Buffers, and Staining Schedules

DOUGLAS E. SOLTIS*, CHRISTOPHER H. HAUFLER**, DAVID C. DARROW***, and GERALD J. GASTONY***

The homosporous pteridophytes have been largely uninvestigated by electrophoresis, despite the fact that they offer many exciting research possibilities (Soltis et al., 1980). The paucity of electrophoretic studies of ferns and fern allies may be due in large part to the high concentrations of condensed tannins that many species contain (Cooper-Driver, 1976 and pers. comm.). These compounds render enzymes inactive by binding with them following cellular disruption, thereby frustrating researchers who have attempted electrophoretic analysis utilizing standard methods of sample preparation.

The method of sample preparation developed by Kelley and Adams (1977a, b) in their analysis of enzyme variation in *Juniperus* was an important procedural breakthrough in overcoming the difficulties that result from the liberation of large amounts of phenolic compounds during tissue preparation. Recently, a simplified version of that method was applied by Soltis et al. (1980) to fern leaf tissue, facilitating rapid preparation of active enzyme samples and thereby making electrophoretic analyses of large numbers of individuals more feasible.

In an attempt to improve methods of analysis of fern enzymes in starch gel electrophoresis, we have experimented with modifications of the method of sample preparation outlined by Soltis et al. (1980). We also have examined several different methods of sample preparation such as those of Gottlieb (1981a), Mitton et al. (1979), and Werth et al. (1982), and have evaluated the relative merits of each with fern tissue. Finally, during the course of our electrophoretic investigations of ferns we found that standard gel and electrode buffers and staining schedules, such as those of Brewer (1970) and Shaw and Prasad (1970), often provided unsatisfactory results when applied to ferns. We have determined gel and electrode buffers, as well as staining schedules, that provide clear starch gel enzyme banding for 22 enzyme systems in ferns. Requests for advice resulting from the recent surge of interest in fern enzyme electrophoresis have prompted us to compile our procedural data so that other researchers can take advantage of our experimentation. We hope that these data will stimulate more extensive electrophoretic investigation of pteridophytes and other electrophoretically difficult taxa.

Gottlieb (1981b) recently reviewed aspects of enzyme electrophoresis primarily in gymnosperms and angiosperms. His discussion is equally relevant to understanding the potential applications and limitations of electrophoretic evidence in pteridophytes. Since homosporous pteridophytes have high chromosome numbers, it is tempting to invoke polyploidy in interpreting their enzyme band patterns. It is well

*Department of Biology, University of North Carolina, Greensboro, NC 27412.
**Department of Botany, University of Kansas, Lawrence, KS 66045.
***Department of Biology, Indiana University, Bloomington, IN 47405.

TABLE 1. ELECTRODE AND GEL BUFFER RECIPES USED SUCCESSFULLY IN ELECTROPHORETIC ANALYSIS OF FERNS (Gram amounts are given for one liter final volume of buffer except where noted).

Electrode Buffer	pH @ 22°C	*Gel Buffer*	pH @ 22°C
1. 0.400 M Citric Acid, trisodium salt; 117.64 g Citric Acid, trisodium salt dihydrate, 1.0 M HCl to pH 7.0	7.0	0.020 M Histidine·HCl; 4.19 g L-Histidine·HCl monohydrate, 1.0 M NaOH to pH 7.0	7.0
2. 0.135 M Tris, 0.032 M Citric Acid; 16.35 g Tris[a], 6.10 g Citric Acid[b]	8.0	0.009 M Tris, 0.002 M Citric Acid; dilute 67 ml of electrode buffer to 1 liter	8.0[c]
3. 0.135 M Tris, 0.017 M Citric Acid; 16.35 g Tris[a], 3.35 g Citric Acid[b]	8.5	0.009 M Tris, 0.001 M Citric Acid; dilute 67 ml of electrode buffer to 1 liter	8.5[c]
4. 0.223 M Tris, 0.086 M Citric Acid; 27.00 g Tris[a], 16.52 g Citric Acid[b], NaOH to pH 7.5	7.5	0.008 M Tris, 0.003 M Citric Acid; dilute 35 ml of electrode buffer to 1 liter	7.5[c]
5. 0.223 M Tris, 0.069 M Citric Acid; 27.00 g Tris[a], 13.33 g Citric Acid[b]	7.2	0.008 M Tris, 0.002 M Citric Acid; dilute 35 ml of electrode buffer to 1 liter	7.2[c]
6. 0.100 M NaOH, 0.300 M Boric Acid; 4.00 g NaOH, 18.55 g Boric Acid[b]	8.6	0.015 M Tris, 0.004 M Citric Acid; 1.84 g Tris[a], 0.69 g Citric Acid[b]	7.8
7. 0.038 M LiOH, 0.188 M Boric Acid; 1.60 g $LiOH \cdot H_2O$, 11.60 g Boric Acid[b] Adjust to pH 8.3 with dry components	8.3	0.045 M Tris, 0.007 M Citric Acid, 0.004 M LiOH, 0.019 M Boric Acid; Tris-citrate buffer (5.45 g Tris[a], 1.28 g Citric Acid[b], bring volume to 900 ml), add 100 ml electrode buffer to give 9:1 ratio, 1.0 M NaOH to pH 8.3	8.3

8. 0.039 M LiOH, 0.263 M Boric Acid; 1.64 g $LiOH \cdot H_2O$, 16.23 g Boric Acid[b]	8.0	0.042 M Tris, 0.007 M Citric Acid; 0.004 M LiOH, 0.025 M Boric acid; 5.04 g Tris[a], 1.25 g Citric Acid[b], 0.16 g $LiOH \cdot H_2O$, 1.56 g Boric Acid[b], 1.0 M HCl to pH 7.6	7.6 7.6
9. 0.065 M L-Histidine free base, ca. 0.015 to 0.016 M Citric Acid; 10.09 g L-Histidine free base, Citric Acid[b] to pH 5.7 (= ca. 2.9 to 3.1 g)	5.7	0.009 M L-Histidine, 0.002 M Citric Acid; dilute 140 ml of electrode buffer to 1 liter	5.7
10. 0.180 M Tris, 0.004 M EDTA, 0.100 M Boric Acid; 21.80 g Tris[a], 1.52 g EDTA tetrasodium salt dihydrate, 6.18 g Boric Acid[b], Boric Acid to pH 8.6	8.6	0.045 M Tris, 0.001 M EDTA, 0.025 M Boric Acid; dilute 250 ml of electrode buffer to 1 liter	8.6
11. 0.400 M Citric Acid, trisodium salt; 117.64 g Citric Acid, trisodium salt dihydrate, 1.0 M HCl to pH 7.0	7.0	0.005 M Histidine·HCl; 1.05 g L-Histidine·HCl monohydrate, 1.0 M NaOH to pH 7.0	7.0

[a]Trizma-base (Sigma T1503; Sigma T1378 works as well and is much less expensive).
[b]Amounts of Citric Acid and Boric Acid are given in grams of anhydrous free acid per liter.
[c]Check pH of solution after mixing; pH of Tris buffers will change with dilution.

Buffer systems 1 and 11 are from Gottlieb (1981a).
Buffer systems 2 and 3 are modifications of buffer system I of Shaw and Prasad (1970).
Buffer systems 4 and 5 are modifications of buffer system XII of Shaw and Prasad (1970).
Buffer system 6 is from Mitton et al. (1977).
Buffer system 7 is a modification of the buffer system of Gottlieb (1981a).
Buffer system 8 is a modification of the buffer system of Adams and Joly (1980).
Buffer systems 9 and 10 are from Gottlieb (pers. comm.).

known, however, that multiple forms of a given enzyme may be coded by different alleles at a single locus (allozymes) or by genes at more than one locus (isozymes). Furthermore, many standardly assayed plant enzymes have isozymes located in two or more subcellular compartments (e.g., the cytosolic and chloroplastic isozymes of PGI and PGM discussed by Gottlieb, 1981b, 1982—for interpretation of enzyme symbols see *Table 2*). When found in pteridophytes, multiple, subcellularly compartmentalized isozymes should not be misinterpreted as products of duplicated loci resulting from polyploidy.

According to Chapman et al. (1979), attempts to determine the amount of heterozygosity per locus in homosporous pteridophytes are complicated by recombination between homoeologous duplicated loci. An obvious prerequisite to recombination of this kind is the actual presence of such loci. Gastony and Gottlieb (1982) developed a means of demonstrating whether duplicated loci are, in fact, present in pteridophytes. By electrophoretically analyzing sporophytes taken from nature and individual gametophytes grown from spores of these sporophytes, segregational analysis of sporophytic enzyme banding patterns can be conducted. This permits genetic interpretation of parental sporophytic enzyme phenotypes without the time-consuming crossing programs required by total reliance on sporophytic tissue. Use of this methodology enables the investigator to determine whether apparent heterozygosity of sporophytes is coded by alleles at a single locus or by genes at duplicated (homoeologous) loci. Our application of this methodology to several fern genera (e.g., *Athyrium*, *Bommeria*, and *Pellaea*) has demonstrated that the genetic variability observed in these taxa results from allelic diversity and segregation at single, not duplicated, loci.

MATERIALS

Living sporophytes of *Athyrium filix-femina*, *Bommeria ehrenbergiana*, *B. hispida*, *B. pedata*, *B. subpaleacea*, *Botrychium virginianum*, *Ceratopteris thalictroides*, *Cystopteris bulbifera*, *C. dickieana*, *C. fragilis*, *C. laurentiana*, *C. protrusa*, *C. reevesiana*, *C. tennesseensis*, *C. tenuis*, *Isoëtes butleri*, *I. engelmannii*, *Lygodium japonicum*, *Nephrolepis exaltata*, *Ophioglossum engelmannii*, *Pellaea andromedifolia*, *P. atropurpurea*, *P. glabella*, *Polypodium polypodioides*, *P. virginianum*, *Polystichum acrostichoides*, *Pteridium aquilinum*, *Woodsia obtusa*, and *W. oregana* were maintained in greenhouse culture and utilized in this investigation. Living gametophytes representing the species of *Athyrium*, *Bommeria*, *Cystopteris*, *Pellaea* and *Pteridium* listed above were cultured on nutrient agar (Gastony & Haufler, 1976) and also provided material for this study.

GRINDING BUFFER SOLUTIONS

We routinely utilize modifications of either the phosphate grinding buffer–polyvinylpyrrolidone (PVP) solution employed by Mitton et al. (1979), the Tris–maleate grinding buffer–PVP solution of Soltis et al. (1980), or the Tris–HCl grinding buffer–PVP solution of Gottlieb (1981a) with PVP substituted for polyvinylpolypyrrolidone (PVPP), as described below. Recipes for preparing these grinding buffer solutions are provided below; molarity or percent volume values are provided and

gram or milliliter amounts required to prepare 25 ml of buffer solution are given in parentheses.

Phosphate grinding buffer–PVP solution.—0.029 M (0.28 g) sodium tetraborate, 0.017 M (0.08 g) sodium metabisulfite, 0.20 M (1.0 g) L-ascorbic acid sodium salt, 0.016 M (0.07 g) diethyldithiocarbamic acid sodium salt, 4% w/v (1.0 g) PVP average molecular weight 40,000 (Sigma PVP 40T), or 36–40% w/v (9.0–10.0 g) PVP average molecular weight 10,000. Dissolve gram amounts in 25 ml of 0.10 M phosphate buffer pH 7.5 (to make 100 ml of phosphate buffer dissolve 1.36 g KH_2PO_4 in H_2O, add 9.0 ml 1M NaOH, and bring volume to 100 ml with H_2O) and then add 0.25 ml (1%) 2-mercaptoethanol.

Tris–maleate grinding buffer–PVP solution.—0.20 M (1.91 g) sodium tetraborate, 0.02 M (0.095 g) sodium metabisulfite, 0.25 M (1.24 g) L-ascorbic acid sodium salt, 0.026 M (0.113 g) diethyldithiocarbamic acid sodium salt, 0.10 M (0.29 g) maleic acid, 0.10 M (0.30 g) Tris, 4% w/v (1.0 g) PVP average molecular weight 40,000 or 32–40% w/v (8.0–10.0 g) PVP average molecular weight 10,000. For 25 ml of buffer, dissolve amounts indicated in 19 ml distilled water; mix thoroughly and crush out lumps; adjust to pH 7.5 with 1.0 M HCl; add 0.025 ml (0.1%) 2-mercaptoethanol; add H_2O to 25 ml. Originally (when we utilized PVP 10,000) it was necessary to allow the PVP to hydrate overnight before using the grinding buffer solution. When employing PVP 40,000, it is possible to prepare the grinding buffer solution immediately prior to sample preparation by stirring the PVP into solution.

Tris–HCl grinding buffer–PVP solution.—0.1% v/v (0.025 ml) 2-mercaptoethanol, 0.001 M (0.010 g) EDTA (tetrasodium salt), 0.010 M (0.019 g) potassium chloride, 0.010 M (0.050 g) magnesium chloride hexahydrate, 4 or 20% w/v (1 or 5 g) PVP 40,000, 25 ml 0.10 M Tris–HCl buffer, pH 7.5. Stir the PVP into solution or allow it to hydrate in the buffer overnight.

TABLE 2. GEL AND ELECTRODE BUFFER SYSTEMS THAT WE HAVE FOUND TO YIELD THE BEST BANDING IN FERNS WE HAVE ASSAYED.

Enzyme	*Symbol*	*Gel and Electrode Buffer System* (from Table 1)
Acid phosphatase	APH	6, 7
Aconitase	ACN	1, 5, 9
Aldolase	ALD	1–5, 7, 10, 11
Aspartate aminotransferase	AAT (or GOT)	6, 7, 8
Catalase	CAT	4, 7, 8, 10
Esterase (Colorimetric)	EST	6, 7
Esterase (Fluorescent)	FE	8
Fructose-1,6-diphosphatase	F1,6DP	1, 11
Glucose-6-phosphate dehydrogenase	G6PDH	4, 5, 6, 7
Glutamate dehydrogenase	GDH	3, 5, 7
Glyceraldehyde-3-phosphate dehydrogenase	G3PDH	1, 11
Hexokinase	HK	2, 3, 5, 11
Isocitrate dehydrogenase	IDH	1, 2, 3, 4, 5
Leucine aminopeptidase	LAP	3, 7, 8
Malate dehydrogenase	MDH	1, 4, 5, 9
Malic enzyme	ME	7, 10
Peroxidase	PER	2, 7
Phosphoglucoisomerase	PGI	5, 6, 7
Phosphoglucomutase	PGM	3, 5, 6, 7, 8
6-Phosphogluconate dehydrogenase	6-PGD	1, 2, 4, 5
Shikimate dehydrogenase	SkDH	1, 2, 4, 5, 11
Triosephosphate isomerase	TPI	2, 5, 6, 7, 8

Comparison of these three grinding buffer solutions indicates that for most enzymes the staining results are highly comparable. For some enzymes, however, the Tris–HCl–PVP solution seems to improve enzyme band clarity (e.g., PGI, PGM, LAP), while for other enzymes the reverse is true. In *Pellaea andromedifolia*, for example, PGI banding was sharp with the Tris–HCl–PVP solution but was inhibited by the presence of ascorbic acid and sodium tetraborate in the Tris–maleate–PVP buffer. In *Athyrium filix-femina*, however, the Tris–maleate–PVP solution gives superior banding for PGI and one more observable EST locus when compared to the Tris–HCl–PVP solution. The phosphate–PVP solution often resulted in reduced APH activity when compared to the other two grinding buffer solutions.

In working with species of *Asplenium*, Werth et al. (1982) used a method of sample preparation that uses caffeine, but not PVP. Following this technique, equal weights of tissue and caffeine were ground in a 0.1 M HEPES pH 7.0 buffer with 0.2% 2-mercaptoethanol and 0.5% sodium metabisulfite to produce a slurry. We found that for virtually all enzyme systems investigated, the method of Werth et al. provided results roughly comparable to those obtained with grinding buffers containing PVP. The presence of 0.5% sodium metabisulfite in the grinding buffer appears to be of great importance. When this ingredient is omitted from the buffer, activity is noticeably reduced or almost totally lost for many enzymes. Significantly, the full complement of PGI enzyme bands obtained with PVP was not expressed in most of the fern taxa investigated when sodium metabifsulfite was omitted. These results are in agreement with similar observations of Werth et al. (1982).

It should be emphasized that to obtain the best results, the appropriate amount of PVP depends upon the taxon under investigation and in part upon the molecular weight of the PVP employed. Soltis et al. (1980) utilized a grade of PVP with an average molecular weight of 10,000 (although this is not stated in their report) and incorporated 40% w/v PVP (4 g PVP in 10 ml of Tris–maleate grinding buffer). At present, we routinely employ a grade of PVP with an average molecular weight of 40,000. Both molecular weights of PVP are suitable, but less PVP 40,000 is required than PVP 10,000. The amount of PVP used is an important consideration because, as reported by Soltis et al. (1980), use of excessive amounts of PVP in the preparation of grinding buffer-PVP solutions frequently results in a decrease or complete loss of enzyme activity. Although PVPP is effective with a wide range of angiosperms, its substitution for PVP failed to produce banding for PGI in *Pellaea andromedifolia* whether the PVPP was hydrated in the grinding buffer or added directly to the leaf tissue during grinding.

This discussion stresses the importance of selecting optimal grinding buffer components and procedures for the taxon under investigation. As noted above, certain components of these buffers may inhibit band expression, whereas in other cases the full complement of components may be required to obtain clear banding. We have found that LAP activity is greater if sodium ascorbate, sodium tetraborate, and sodium metabisulfite are eliminated from the grinding buffer. On the other hand, activity for some enzymes, (e.g., SkDH, G6PDH, MDH, CAT, 6PGD) may be very noticeably reduced when a buffer solution incorporating only PVP is

utilized. A further complication in some taxa is that when a grinding buffer lacking 2-mercaptoethanol is employed, artifactual ("ghost") bands proliferate for some enzymes and can hinder accurate interpretation of the band patterns. Given these examples, we strongly encourage comparative experimentation with grinding buffer solutions when initiating electrophoretic studies.

SAMPLE PREPARATION AND ELECTROPHORESIS

Leaf samples to be analyzed electrophoretically were taken from mature sporophytes. The methods discussed herein, however, are applicable to gametophytic as well as sporophytic tissue. In our original electrophoretic analyses of ferns, leaf samples were prepared according to the method of Soltis et al. (1980) in which small amounts of leaf tissue are ground under liquid nitrogen with a porcelain mortar and pestle until a fine dry powder is obtained. The powder is quickly mixed with grinding buffer to form a thick slurry. This slurry is absorbed directly into thick paper wicks (made from Whatmann 3 MM chromatography paper or some other suitable wick paper).

It should be emphasized that the use of the grinding buffer–PVP solutions alone (outlined above), without the use of liquid nitrogen in the preparation of samples, provide clear enzyme banding in all of the fern taxa that we have examined so far. Therefore, at present we utilize a simplified method of sample preparation in which liquid nitrogen has been eliminated. In standardizing our grinding procedure by consistently homogenizing 100 mg of leaf material in 0.5 ml of grinding buffer, we obtained roughly equivalent staining intensity for all samples, which facilitates comparing individuals and scoring gels. If small amounts of tissue are being ground, it may be possible to substitute 43 mm^2 plastic weigh boats for mortars and glass or plexiglass rods for pestles. As discussed by Gastony and Gottlieb (1982), it is possible to obtain enzyme banding from single gametophytes. Their technique involves smearing individual gametophytes onto wicks pre-moistened with extraction buffer–PVP solution. With all of the described grinding/extraction procedures, the amount of grinding buffer and the type and size of wicks should be tailored to the taxon and tissues under investigation. Once the plant material has been absorbed into wicks, the wicks are inserted into a vertical slit in the starch gel and subjected to horizontal electrophoresis at 4°C. Band definition may be improved by removing wicks from the gel after the first 10–20 minutes of electrophoresis. It is important to maintain electrical contact across the slit in the gel. We routinely place some sort of spacer (e.g., strips of Whatmann paper, a plastic straw, a thin piece of plexiglass) at the cathodal end of the gel to compress the gel slightly and to insure that the slit does not open.

To permit comparison of banding patterns between gels, it is important to maintain a constant starch concentration throughout a study. We have experimented with a variety of starch concentrations throughout the practical range of 11.5 to 15% and have found concentrations of 12 to 13.2% most suitable, although in some instances alternative concentrations may improve band resolution. Connaught, Fisher, and Sigma starch have been used and all provide clear enzyme banding, provided appropriate gel and electrode buffer systems and staining schedules are utilized.

In order to facilitate determining the location of the anodal front, it is useful to insert a wick bearing the marker dye Bromphenol Blue (Sulfone Form, Sigma B0126; 0.04% w/v in 95% ethanol). Depending on the gel and electrode buffer system employed, as well as the taxon under investigation, the enzyme bands will migrate varying distances behind the Bromphenol Blue marker. Optimal running times for each enzyme system will therefore have to be determined empirically.

GEL AND ELECTRODE BUFFER RECIPES

We have found that standard gel and electrode buffer systems, such as those of Shaw and Prasad (1970), and Brewer (1970), often yield unsatisfactory results with fern tissue. We have determined gel and electrode buffer systems that provide clear banding for 22 enzymes in ferns. Recipes for the gel and electrode buffers we most commonly employ are provided in *Table 1*.

Several of the gel and electrode buffer systems that we employ are modifications of those of Shaw and Prasad (1970). It should be noted, therefore, that errors are present in several of the recipes they provided. For example, in Shaw and Prasad buffer system I (see page 299, Table 1 of their report), utilization of 16.35 g of Tris yields an electrode buffer with a molarity of 0.135 (see present report, *Table 1*, electrode buffers 2 and 3), rather than 0.155 as reported by Shaw and Prasad. Similarly, use of 27.0 g of Tris in the preparation of the electrode buffer of Shaw and Prasad system XII yields a molarity of not 0.233 but 0.223 (see present report, *Table 1*, electrode buffers 4 and 5). Furthermore, use of the electrode buffer recipe of Shaw and Prasad system XII yields a buffer of approximately pH 5.5, which differs significantly from the estimate of pH 7.0 listed by Shaw and Prasad. Use of the amounts of Tris and citric acid given for system 4 in *Table 1* of the present report will also yield an electrode buffer of pH 5.5; sodium hydroxide is then added to adjust the buffer to pH 7.5.

Of the 11 gel and electrode buffer systems for which recipes are provided in *Table 1*, some clearly are preferable to others for certain of the 22 enzymes for which we have obtained sharp banding. For example, best enzyme banding for MDH is obtained when system 1, 4, 5, or 9 is used. Which system works best is dependent, in part, on the taxon under investigation. We have found, for example, that the best systems for *Athyrium* and *Cystopteris* differ somewhat from those that supply clear enzyme banding for *Bommeria*. The systems provided here, however, should provide an excellent framework for conducting electrophoretic analyses of most ferns. The gel and electrode buffer systems that we have found to yield the clearest banding for each of the 22 enzymes with which we have experimented are listed in *Table 2*.

STANDARD STAINING SCHEDULES

During the course of our electrophoretic investigations of ferns, we found that many published staining schedules, such as those of Shaw and Prasad (1970), yielded unsatisfactory results. Enzyme banding often can be improved dramatically when these standard recipes are modified. One very important modification that we employ involves increasing the pH of the buffer in staining schedules for ALD, GDH, G6PDH, HK, IDH, MDH, ME, PGI, PGM, and 6PGD. Tris–HCl staining buffers of pH 7.0 or 7.1 typically are used in staining for these enzymes (Shaw &

Prasad, 1970). We have found, however, that in ferns the pH optima for these enzymes are in the 8.0–8.5 range, and we therefore use a Tris–HCl staining buffer of pH 8.0–8.5 for them. For some enzymes, this simple modification improves staining dramatically. For example, standard MDH staining recipes use a buffer of pH 7.0. Application of such a protocol to ferns, however, often results in very little or inconsistent staining. Well stained bands are obtained consistently for MDH when a Tris–HCl staining buffer of pH 8.0–8.5 is used (provided an appropriate gel and electrode buffer system is employed; see *Table 2*).

It also should be noted that we have consistently been unable to obtain observable activity with fern leaf tissue for some enzymes, despite considerable experimentation. These include Alcohol dehydrogenase, Alkaline phosphatase, α-Glycerophosphate dehydrogenase and Lactate dehydrogenase. There are several enzymes for which we have obtained observable activity with ferns, but have not yet obtained clear banding, such as Diaphorase and Peptidase, and these are therefore not included in this report. In addition, there are a number of enzyme staining schedules that we have not yet attempted. We hope that the staining schedules provided below will stimulate additional experimentation in this regard.

The staining schedules that we utilize for the 22 enzymes for which we obtain clear banding are provided below in alphabetical order. The final volume for all stain recipes is 100 ml. Depending on gel size and staining container capacity, it may be possible to assay two gel slices simultaneously with each of the following 22 solutions. Alternatively, when assaying single gel slices, the recipes may be halved to 50 ml final volume. Readers are encouraged to consult *Enzyme Nomenclature 1978* (International Union of Biochemistry, Nomenclature Committee, 1979 [hereafter referred to as I.U.B., 1979]) for details of enzyme specificity and Gottlieb (1981b) for a recent review of the electrophoretic technique and its application to plant populations. It should be noted that enzymes such as peroxidases, esterases, and acid phosphatases operate on a large number of substrates in vitro; therefore, standard staining protocols for these enzymes result in the staining of various numbers of isozymes whose homologies are not apparent. Although the utility of these isozymes in between-species comparisons is therefore limited, their banding patterns can be useful in assessing variability within populations.

Several different organisms serve as the source for commercial preparations of Glucose-6-phosphate dehydrogenase. This enzyme typically is specific for NADP, but some forms are capable of utilizing either NADP or NAD. It should be emphasized that NADP is much more expensive than NAD, hence selecting a Glucose-6-phosphate dehydrogenase type that can use NAD will save considerable money. Therefore, in those recipes that call for added Glucose-6-phosphate dehydrogenase, the notation NAD(P)* reflects this option.

Acid phosphatase (EC 3.1.3.2)

0.05 M sodium acetate buffer, pH 5.0	100 ml
1.0 M $MgCl_2$	0.5 ml
α-naphthyl acid phosphate, sodium salt	100 mg
Fast garnet GBC salt	80 mg

We have obtained results with buffer molarities ranging from 0.05 M to 0.2 M and within a pH range of 5.0 to 6.0. Stain at room temperature; a modification of

Scandalios (1969). Gottlieb (1981b) noted that plants may have a dozen or more acid phosphatases. We consistently have observed one very intense zone of acid phosphatase activity, with several fainter bands occasionally evident.

Aconitase (EC 4.2.1.3.; = Aconitate hydratase in I.U.B., 1979)

1.0 M Tris-HCl buffer, pH 8.5	10 ml
H_2O	90 ml
cis-aconitic acid	70 mg
Isocitrate (= Isocitric) dehydrogenase	7 units
1.0 M $MgCl_2$	1 ml
NADP	10 mg
MTT	5 mg
PMS	1 mg

Stain in the dark at 30°C; a modification of Shaw and Prasad (1970).

Aldolase (EC 4.1.2.13; = Fructose-biphosphate aldolase in I.U.B., 1979)

1.0 M Tris-HCl buffer, pH 8.5	10 ml
H_2O	90 ml
Fructose-1,6-diphosphate,	
tetra(cyclohexylammonium) salt	500 mg
or	
trisodium salt	200 mg
1.0 M arsenic acid, sodium salt	1 ml
Glyceraldehyde-3-phosphate dehydrogenase	200 units
NAD	20 mg
MTT	20 mg
PMS	5 mg

Stain at room temperature; a modification of Shaw and Prasad (1970).

Aspartate aminotransferase (EC 2.6.1.1.; = Glutamate oxaloacetate transaminase)

1.0 M Tris-HCl buffer, pH 8.0	10 ml
H_2O	90 ml
L-aspartic acid	100 mg
α-ketoglutaric acid	100 mg

Adjust pH to 8.0 with 1.0 M NaOH as necessary, then add:

Pyridoxal-5′-phosphate	5 mg
Fast blue BB salt	100 mg

Stain in the dark at room temperature; a modification of Gottlieb (1973a) and Selander et al. (1971, appendix). Our results show that this enzyme is very sensitive to the pH of the staining buffer; very little staining is observed at pH 7.0 or at pH 9.0. The number of loci coding for proteins that are capable of this aminotransferase reaction is known to vary in plants. Although we typically encounter a single locus, Gottlieb (1981b) has reported the likelihood of specific subcellular localizations for the several dimeric isozymes.

Catalase (EC 1.11.1.6)

3% H_2O_2	5 ml
0.1 M phosphate buffer, pH 7.0	10 ml
0.06 M $Na_2S_2O_3 \cdot 5H_2O$	7 ml
H_2O	78 ml

Incubate in this solution at room temperature for 1–2(30) minutes; pour off the solution, rinse several times with distilled water, then add:

0.09 M KI	50 ml
H_2O	50 ml

Catalase activity will appear as white bands on a dark blue background; a modification of Shaw and Prasad (1970). For some of the buffer systems in *Table 1*, it may be necessary to add several drops (approximately 2 ml) of glacial acetic acid in order to induce staining. An alternative method is to substitute 50 ml 0.05 M sodium acetate buffer (pH 5.0) for the 50 ml H_2O. The gel may turn completely blue in a very short time. Be prepared either to photograph the gel or score it while staining. One locus has been observed in ferns. The protein is reported to be tetrameric (Scandalios, 1969).

Esterase (Colorimetric; EC 3.1.1.–)

α-naphthyl acetate	40 mg
ß-naphthyl acetate	40 mg
dissolved in acetone	2 ml
1.0 M phosphate buffer, pH 6.0	10 ml
H_2O	90 ml
Fast blue RR salt	100 mg

Stain at room temperature; a modification of Gottlieb (1974).

Esterase (Fluorescent; EC 3.1.1.–)

4-methylumbelliferyl acetate	42 mg
dissolved in acetone	25 ml
1.0 M sodium acetate buffer, pH 5.0	18 ml
H_2O	57 ml

Stain in the dark at room temperature and observe under long-wave ultraviolet light; staining schedule of Mitton et al. (1979). Observe immediately after staining (bands fade quickly). Caution should be exercised in scoring gels because flavonoid "bands" may also be visualized under UV light. To determine whether flavonoid "bands" are present, the gel slice should be observed under UV light prior to staining.

Fructose-1,6-diphosphatase (EC 3.1.3.11; =Fructose-biphosphatase in I.U.B., 1979)

1.0 M Tris-HCl buffer, pH 8.0	10 ml
H_2O	90 ml
Fructose-1,6-diphosphate, tetra(cyclohexylammonium) salt	250 mg
or	
trisodium salt	100 mg
1.0 M $MgCl_2$	1 ml
Phosphoglucoisomerase	50 units
Glucose-6-phosphate dehydrogenase	50 units
NAD(P)*	10 mg
MTT	5 mg
PMS	1 mg

Stain in the dark at 30° C; a modification of Shaw and Prasad (1970).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

1.0 M Tris-HCl buffer, pH 8.0 or 8.5	10 ml
H_2O	90 ml
Glucose-6-phosphate, disodium salt	100 mg
NADP	20 mg
MTT (or NBT)	10 mg
PMS	2 mg

Stain in the dark at 37° C; a modification of Shaw and Prasad (1970).

Glutamate dehydrogenase (EC 1.4.1.2)

1.0 M Tris-HCl buffer, pH 8.0	10 ml
H_2O	70 ml
1.0 M L-glutamic acid, pH 8.0 (use free acid or monosodium salt and add NaOH to pH 8.0)	20 ml
NAD	20 mg
MTT (or NBT)	10 mg
PMS	2 mg

Stain in the dark at room temperature; a modification of Gottlieb (1973b) and of Shaw and Prasad (1970).

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; = Glyceraldehyde-phosphate dehydrogenase in I.U.B., 1979)

1.0 M Tris-HCl buffer, pH 8.0	10 ml
H_2O	90 ml
Fructose-1,6-diphosphate, trisodium salt	100 mg
Aldolase	10 units

Incubate above mixture approx. 30 min. at 30–37° C, then add:

1.0 M arsenic acid, sodium salt	1 ml
NAD	10 mg
MTT	5 mg
PMS	1 mg

Stain in the dark at 30° C; a modification of Shaw and Prasad (1970).

Hexokinase (EC 2.7.1.1)

1.0 M Tris–HCl buffer, pH 8.5	10 ml
H_2O	90 ml
Glucose	90 mg
1.0 M $MgCl_2$	5 ml
EDTA, tetrasodium salt, dihydrate	40 mg
NAD(P)*	10 mg
MTT (or NBT)	15 mg
Glucose-6-phosphate dehydrogenase	40 units
PMS	2 mg
ATP	25 mg

Stain in the dark at room temperature; a modification of Shaw and Prasad (1970).

Isocitrate dehydrogenase [$NADP^+$] (EC 1.1.1.42)

1.0 M Tris–HCl buffer, pH 8.0	10 ml
H_2O	85 ml
Isocitric acid, trisodium salt	100 mg
1.0 M $MgCl_2$	5 ml
NADP	10 mg

MTT (or NBT)	15 mg
PMS	2 mg

Depending on the taxon being analyzed, results have been obtained by using buffers ranging from pH 7.2 to pH 8.5. Stain in the dark at room temperature; a modification of Shaw and Prasad (1970).

Leucine aminopeptidase (EC 3.4.11.–)

L-leucine-β-naphthylamide (free base or acid salt)	20 mg
dissolved in dimethyl formamide	5 ml
1.0 M phosphate buffer, pH 6.0	10 ml
H_2O	90 ml
Black K salt or fast black K salt	50 mg

Stain in the dark at room temperature; a modification of Gottlieb (1973c). This enzyme has broad activity; it can cleave a number of N-terminal amino acids. Although commonly referred to as Leucine aminopeptidase ("LAP"), it is more precisely referred to as Aminopeptidase. There are a number of aminopeptidases, some of which are specific for certain N-terminal amino acids. Aminopeptidase (cytosol; EC 3.4.11.1) is activated by heavy metals, whereas Aminopeptidase (microsomal; EC 3.4.11.2) is not activated by heavy metals (I.U.B., 1979). It may be necessary to modify the pH of the stain in order to increase staining intensity. One isozyme is usually observed and a second is occasionally apparent; both are monomeric.

Malate dehydrogenase (EC 1.1.1.37)

1.0 M Tris-HCl buffer, pH 8.0 or 8.5	10 ml
2.0 M DL-malic acid, pH 8.0 (add NaOH to pH 8.0)	10 ml
H_2O	80 ml
NAD	10 mg
MTT (or NBT)	10 mg
PMS	2 mg

Stain in the dark at room temperature; a modification of Shaw and Prasad (1970). Some investigators add 38 mg EDTA (tetrasodium salt, dihydrate) to this recipe. We use 2.0 M DL-malic acid rather than 1.0 M L-malic acid as most standard staining schedules require because DL-malic acid is much less expensive than purified L-malic acid, which is the actual substrate. There are at least four malate NAD^+ dehydrogenases reported (I.U.B., 1979). The one most frequently reported in routine electrophoresis is L-Malate: NAD^+ oxidoreductase (EC 1.1.1.37), of which at least two putative isozymes have been observed. The protein is dimeric; subcellular compartmentalization of the isozymes is discussed by Gottlieb (1981b).

Malic enzyme (EC 1.1.1.40; = Malate dehydrogenase [Oxaloacetate-decarboxylating, $NADP^+$] in I.U.B., 1979)

1.0 M Tris-HCl buffer, pH 8.0 or 8.5	10 ml
H_2O	80 ml
2.0 M DL-malic acid, pH 8.0 (prepare as in Malate dehydrogenase)	10 ml
1.0 M $MgCl_2$	2 ml
NADP	20 mg
MTT (or NBT)	20 mg
PMS	2 mg

Stain in the dark at room temperature; a modification of Richmond (1972 and pers. comm.).

Peroxidase (EC 1.11.1.7)

3-amino-9-ethyl carbazole	65 mg
dissolved in dimethyl formamide	5 ml
0.05 M sodium acetate buffer, pH 5.0	95 ml
0.1 M $CaCl_2$	2 ml
3% H_2O_2	2 ml

Incubate the gel in a refrigerator until the bands appear (30–60 min); a modification of Shaw and Prasad (1970) and of Gottlieb (1973b).

Phosphoglucoisomerase (EC 5.3.1.9; = Glucosephosphate isomerase in I.U.B., 1979)

1.0 M Tris–HCl buffer, pH 8.0	10 ml
H_2O	90 ml
1.0 M $MgCl_2$	1 ml
Fructose-6-phosphate, disodium salt	30 mg
Glucose-6-phosphate dehydrogenase	40 units
NAD(P)*	10 mg
MTT (or NBT)	20 mg
PMS	2 mg

Stain in the dark at 37°C or at room temperature; a modification of Shaw and Prasad (1970). This dimeric enzyme has numerous synonyms (I.U.B., 1979) including: Phosphohexose isomerase, Phosphohexomutase, Oxoisomerase, Hexose phosphate isomerase, Glucose-6-phosphate isomerase, Phosphosaccharomutase, and Phosphohexoisomerase. Cytosolic and chloroplastic isozymes should be expected (Gottlieb, 1981b, 1982).

Phosphoglucomutase (EC 2.7.5.1)

1.0 M Tris–HCl buffer, pH 8.0 or 8.5	10 ml
H_2O	90 ml
1.0 M $MgCl_2$	2 ml
Glucose-1-phosphate, disodium (Sigma G7000) or dipotassium (Sigma G6875) salt	100 mg
1.7×10^{-4} M α-D-glucose-1,6-diphosphate, tetra(cyclohexylammonium) salt	5 ml
Glucose-6-phosphate dehydrogenase	40 units
NAD(P)*	10 mg
MTT (or NBT)	20 mg
PMS	2 mg

A modification of Shaw and Prasad (1970). An equally suitable alternate recipe which eliminates the need to purchase α-D-glucose-1,6-diphosphate is:

1.0 M Tris–HCl buffer, pH 8.0	10 ml
H_2O	90 ml
1.0 M $MgCl_2$	2 ml
Glucose-1-phosphate, disodium salt (Sigma G1259)	50 mg
Glucose-6-phosphate dehydrogenase	40 units
NAD(P)*	10 mg
MTT	10 mg
PMS	2 mg

Stain in the dark at 37°C or at room temperature.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44)

1.0 M Tris–HC1 buffer, pH 8.0	10 ml
H_2O	90 ml
6-phosphogluconic acid, barium salt	40 mg
1.0 M $MgCl_2$	2 ml
NADP	10 mg
MTT	10 mg
PMS	2 mg

Stain in the dark at room temperature or at 37°C; a modification of Shaw and Prasad (1970).

Shikimate dehydrogenase (EC 1.1.1.25)

1.0 M Tris–HC1 buffer, pH 8.5	10 ml
H_2O	90 ml
Shikimic acid	100 mg
NADP	10 mg
MTT (or NBT)	20 mg
PMS	2 mg

Stain in the dark at room temperature or at 37°C.

Triosephosphate isomerase (EC 5.3.1.1)

1.0 M Tris-HC1 buffer, pH 8.0	10 ml
H_2O	90 ml
Dihydroxyacetone phosphate, lithium salt	10 mg
EDTA, tetrasodium salt, dihydrate	38 mg
NAD	30 mg
MTT	10 mg
PMS	2 mg
Arsenic acid, sodium salt	460 mg
Glyceraldehyde-3-phosphate dehydrogenase	300 units

Stain in the dark at 37°C; Gottlieb (pers. comm.). An alternative and much less expensive method of staining for TPI follows (also see agarose recipe):

1.0 Tris–HC1 buffer, pH 8.0	10 ml
H_2O	90 ml
DL-α-glycerophosphate	200 mg
Pyruvic acid, sodium salt	100 mg
α-Glycerophosphate dehydrogenase	100 units
Lactate dehydrogenase	100 units
NAD	20 mg

Incubate the above solution for 2 hours at 30–37°C. At the end of the incubation period, inactivate the enzymes by adjusting the solution to pH 2.0 with 1.0 M HC1 (taking care not to go below pH 2.0) and then re-adjust to pH 8.0 with 1.0 M NaOH. Add:

1.0 M arsenic acid, sodium salt	1 ml
Glyceraldehyde-3-phosphate dehydrogenase	100 units
NAD	20 mg
MTT	5 mg
PMS	1 mg

Stain in the dark at 30°C. The bands will be dark blue on a light blue background. Readers who follow this protocol must first show the lack of α-Glycerophosphate dehydrogenase activity in their taxa. This can be done simply by staining a gel slice with:

1.0 M Tris-HCl buffer, pH 8.0	10 ml
H_2O	90 ml
DL-α-glycerophosphate	200 mg
NAD	20 mg
MTT	5 mg
PMS	1 mg

AGAROSE STAINING SCHEDULES

Several of the reagents commonly required in enzyme electrophoresis, such as NADP, are expensive. Costs may be reduced by using staining schedules employing agarose (modified from Mitton et al., 1979 and Gaines, pers. comm.), which require much smaller quantities of most ingredients than do standard staining schedules but yield comparable results.

Agarose staining schedules are provided below for ALD, G6PDH, GDH, IDH, MDH, PGI, PGM, 6-PGD, SkDH, and TPI. The following general procedure is employed to stain one starch gel slice using the agarose technique: dissolve all ingredients required for enzyme staining (see below) in 6 ml of Tris–HCl buffer; in a second flask mix 0.06 g agarose (Agarose type II, Sigma A6877) with 6 ml distilled water (a 1% agarose solution) and bring this solution to a boil while stirring constantly; combine the two solutions and very quickly apply to surface of one gel slice (it may be necessary to increase ml amounts of buffer and 1% agarose depending on the size of the gel slice); stain all slices in the dark at room temperature.

Aldolase (EC 4.1.2.13)

PMS	1 mg
MTT	5 mg
NAD	10 mg
Fructose-1,6-diphosphate, trisodium salt or tetra(cyclohexylammonium) salt	75 mg
Arsenic acid	25 mg
Glyceraldehyde-3-phosphate dehydrogenase	40 units
0.1 M Tris-HCl buffer, pH 8.5	6 ml
1% agarose	6 ml

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

PMS	1 mg
MTT	4 mg
NADP	4 mg
EDTA, tetrasodium salt, dihydrate	6 mg
Glucose-6-phosphate, disodium salt	5 mg
0.1 M Tris–HCl buffer, pH 8.0	6 ml
1% agarose	6 ml

Glutamate dehydrogenase (EC 1.4.1.2)

PMS	1 mg
MTT	4 mg
NAD	3 mg
L-glutamic acid, monosodium salt	100 mg
0.1 M Tris–HCl buffer, pH 8.0	6 ml
1% agarose	6 ml

Isocitrate dehydrogenase [$NADP^+$] (EC 1.1.1.42)

PMS	1 mg
MTT	4 mg
NADP	4 mg
$MgCl_2$	40 mg
Isocitric acid, trisodium salt	10 mg
0.1 M Tris–HCl buffer, pH 8.0	6 ml
1% agarose	6 ml

Malate dehydrogenase (EC 1.1.1.37)

PMS	2 mg
MTT	7 mg
NAD	7 mg
L-malic acid	16 mg
0.25 M Tris–HCl buffer, pH 8.6	6 ml
1% agarose	6 ml

Keep PMS and L-malic acid powders separate until adding the buffer.

Phosphoglucoisomerase (EC 5.3.1.9)

PMS	1 mg
MTT	4 mg
NAD(P)*	1 mg
0.1 M Tris–HCl buffer, pH 8.0	4 ml
10% $MgCl_2$	1 ml
0.018 M fructose-6-phosphate, disodium salt	1 ml
Glucose-6-phosphate dehydrogenase	10 units
1% agarose	6 ml

Phosphoglucomutase (EC 2.7.5.1)

PMS	1 mg
MTT	5 mg
NAD(P)*	4 mg
0.1 M Tris–HCl buffer, pH 8.0	2 ml
0.05 M glucose-1-phosphate, disodium salt (Sigma G7000)	2.5 ml
Glucose-6-phosphate dehydrogenase	10 units
0.001 M fructose-1,6-diphosphate, trisodium salt	1 ml
10% $MgCl_2$	1 ml
1% agarose	6 ml

6-Phosphogluconate dehydrogenase (EC 1.1.1.4.4)

PMS	1 mg
MTT	4 mg
NADP	4 mg
6-phosphogluconic acid, barium salt	10 mg
10% $MgCl_2$	0.5 ml
0.1 M Tris–HCl buffer, pH 8.5	5 ml
1% agarose	6 ml

Dissolve the 6-phosphogluconic acid barium salt in 1.0 ml of 0.1 M Tris–HCl buffer (pH 8.5) approximately 15 minutes before mixing in other ingredients.

Shikimate dehydrogenase (EC 1.1.1.25)

PMS	1 mg
MTT	4 mg
NADP	4 mg
Shikimic acid	10 mg
0.1 M Tris–HC1 buffer, pH 8.0	6 ml
1% agarose	6 ml

Triosephosphate isomerase (EC 5.3.1.1)

PMS	1 mg
MTT	4 mg
NAD	10 mg
EDTA, tetrasodium salt, dihydrate	10 mg
Arsenic acid	150 mg
Dihydroxyacetone phosphate	1.5 mg
Glyceraldehyde-3-phosphate dehydrogenase	75 units
0.1 M Tris–HC1 buffer, pH 8.0	6 ml
1% agarose	6 ml

GEL FIXATION AND DOCUMENTATION

AAT and PER gel slices are fixed in 50% glycerol; all other gel slices are fixed in 50% ethanol.

In order to keep a permanent record of results, we routinely photograph gel slices using Kodak Technical Pan Film 2415 following the exposure index and developing procedure recommended for high contrast.

ACKNOWLEDGEMENTS

D.E.S. and C.H.H. thank Jeff Atwood, Harriet Blanton, Leslie Gottlieb, James Hamrick, Terry Lastovicka, and Andrew Torres for laboratory assistance and helpful comments and Charles Werth for providing details of his sample preparation methodology. G.J.G. is grateful to Leslie Gottlieb for the use of laboratory facilities and advice during work with *Pellaea andromedifolia*. Grants-in-aid of research to D.E.S. from the Research Council of the University of North Carolina at Greensboro, to C.H.H. from General Research Fund Grants 3005 and 3588 of the University of Kansas, and to D.C.D. from the Department of Biology of Indiana University, NSF Doctoral Dissertation Improvement Grant DEB-8117239, and the Society of the Sigma Xi are gratefully acknowledged.

LITERATURE CITED

ADAMS, W. T. and R. J. JOLY. 1980. Genetics of allozyme variants in loblolly pine. J. Hered. 71:33–40.

BREWER, G. J. 1970. An Introduction to Isozyme Techniques. Academic Press, New York.

CHAPMAN, R. H., E. J. KLEKOWSKI, Jr., and R. K. SELANDER. 1979. Homoeologous heterozygosity and recombination in the fern Pteridium aquilinum. Science 204:1207–1209.

COOPER-DRIVER, G. 1976. Chemotaxonomy and phytochemical ecology of bracken. J. Linn. Soc., Bot. 73:35–46.

GASTONY, G. J. and L. D. GOTTLIEB. 1982. Evidence for genetic heterozygosity in a homosporous fern. Amer. J. Bot. 69:634–637.

———, and C. H. HAUFLER. 1976. Chromosomes and apomixis in the fern genus Bommeria (Gymnogrammaceae). Biotropica 8:1–11.

GOTTLIEB, L. D. 1973a. Genetic control of Glutamate oxaloacetate transaminase isozymes in the diploid plant Stephanomeria exigua and its allotetraploid derivative. Biochem. Genet. 9:97–107.

————. 1973b. Genetic differentiation, sympatric speciation, and the origin of a diploid species of Stephanomeria. Amer. J. Bot. 60:545–553.

————. 1973c. Enzyme differentiation and phylogeny in Clarkia franciscana, C. rubicunda and C. amoena. Evolution 27:205–214.

————. 1974. Genetic confirmation of the origin of Clarkia lingulata. Evolution 28:244–250.

————. 1981a. Gene numbers in species of Astereae that have different chromosome numbers. Proc. Natl. Acad. Sci. USA 78:3726–3729.

————. 1981b. Electrophoretic evidence and plant populations. Progress in Phytochemistry 7:1–45.

————. 1982. Conservation and duplication of isozymes in plants. Science 216:373–380.

INTERNATIONAL UNION OF BIOCHEMISTRY, NOMENCLATURE COMMITTEE. 1979. Enzyme Nomenclature 1978. Academic Press, New York.

KELLEY, W. A. and R. P. ADAMS. 1977a. Preparation of extracts from Juniper leaves for electrophoresis. Phytochemistry 16:513–516.

————, and R. P. ADAMS. 1977b. Seasonal variation of isozymes in Juniperus scopulorum: systematic significance. Amer. J. Bot. 64:1092–1096.

MITTON, J. B., Y. B. LINHART, J. L. HAMRICK, and J. S. BECKMAN. 1977. Observations on the genetic structure and mating system of ponderosa pine in the Colorado front range. Theor. Appl. Genet. 51:5–13.

————, Y. B. LINHART, K. B. STURGEON, and J. L. HAMRICK. 1979. Allozyme polymorphism detected in mature needle tissue of ponderosa pine. J. Hered. 70:86–89.

RICHMOND, R. C. 1972. Enzyme variability in the Drosophila willistoni group. II. Amounts of variability in the superspecies, D. paulistorum. Genetics 70:87–112.

SCANDALIOS, J. G. 1969. Genetic control of multiple molecular forms of enzymes in plants: a review. Biochem. Genet. 3:37–79.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, and J. B. GENTRY. 1971. IV. Biochemical polymorphism and systematics in the genus Peromyscus. I. Variation in the old-field mouse (Peromyscus polionotus). Studies in genetics VI. Univ. Texas Publ. 7103:49–90.

SHAW, C. R. and R. PRASAD. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. Biochem. Genet. 4:297–320.

SOLTIS, D. E., C. H. HAUFLER, and G. J. GASTONY. 1980. Detecting enzyme variation in the fern genus Bommeria: an analysis of methodology. Syst. Bot. 5:30–38.

WERTH, C. R., A. A. KARLIN, and S. I. GUTTMAN. 1982. Enzyme extraction from phenolic-containing plant tissues using caffeine. Isozyme Bull. 15:139.